SHOCK, Vol. 13, No. 3, pp. 217–223, 2000

Role of Endogenous Nitric Oxide in TNF-α and IL-1β Generation in Hepatic Ischemia-Reperfusion

Peitan Liu, Baohuan Xu, Eric Spokas, Pi-Shiang Lai, and Patrick Y-K Wong

Department of Cell Biology, University of Medicine and Dentistry of New Jersey, School of Osteopathic Medicine, Stratford, New Jersey 08084

Received 8 Mar 1999; first review completed 30 Mar 1999; accepted in final form 21 Sep 1999

ABSTRACT—In the present study, we examined the role of nitric oxide (NO) in early-response cytokine production by using a rat model of hepatic ischemia-reperfusion (HI/R). The left and median lobes of the liver were subjected to 30 min of ischemia, followed by 4 h of reperfusion. Group I and II rats were sham-operated controls that received saline (vehicle) or N^W-nitro-L-arginine methylester (L-NAME) (10 mg/kg, iv); group III and IV rats were subjected to HI/R and received vehicle or L-NAME (10 mg/kg, iv, 10 min before reperfusion), respectively. Administration of L-NAME to rats subjected to I/R resulted in a fourfold decrease in plasma NO levels, accompanied by a marked increase of plasma alanine aminotransferase (ALT) activity relative to group III. These changes in group IV were associated with elevation of superoxide generation in ischemic liver lobes by 2.1-fold and circulating leukocyte number by 1.42-fold, compared with group III. Normalized for expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger ribonucleic acid (mRNA), expression of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) mRNA in ischemic liver of group IV was augmented by 207% and 175% compared with Group III. The expression of (iNOS) mRNA was also increased (223%) relative to group III. Moreover, in group IV, plasma TNF-α levels at 4 h of reperfusion and IL-1β levels at 90 min and 4 h of reperfusion were significantly increased compared with group III. No statistically significant changes were observed between groups I and II in plasma ALT activity, plasma NO levels, circulating leukocyte counts, superoxide generation in the ischemic lobes of liver, and plasma TNF-α and IL-1β concentrations. The observed enhancement of I/R injury by L-NAME is consistent with the hypothesis that endogenous NO down-regulates TNF-α and IL1β generation, thereby decreasing HI/R injury.

KEYWORDS—ischemia-reperfusion injury, early-response cytokine, L-NAME, reverse transcription-polymerase chain reaction, NOS inhibitor

INTRODUCTION

Nitric oxide (NO) participates in the hypotension associated with sepsis in experimental animals (1–3) and humans (4–6). However, inhibition of NO synthesis may result in detrimental effects (6–9). Administration of inhibitors of NO synthase (NOS) resulted in improved hemodynamics but did not diminish mortality in a rat model of endotoxin shock (10) and in patients with endotoxin shock (6). Inhibition of NO synthesis in patients with septic shock was also associated with pulmonary hypertension and decreased cardiac output (11). On the other hand, for more than a century nitroglycerin (and other NO donors) has been used to treat patients with ischemic cardiovascular diseases.

Effects of "early-response" cytokines (i.e., tumor necrosis factor-α [TNF-α] and interleukin-1β [IL-1β]) and their receptor antagonists (such as IL-1ra and sTNF-R) on inflammation have been intensively investigated (12, 13). It is clear that complement activation is a very early event occurring after restoration of blood flow in models of ischemia-reperfusion (I/R) injury. In particular, C5a binding to a receptor on macrophage membranes acts as a stimulus for the synthesis of new TNF-α and other proinflammatory cytokines by macrophages (14, 15). Subsequently, the release of cytokines is further amplified. When the levels of TNF-α and IL-1β reach the general circulation in sufficient concentrations, they can stimulate systemic inflammation in an endocrine fashion. This amplification process can also extend to vascular endothelial cells in which expression of P-selectin, and ICAM-1 is upregulated (8).

The relationship between NO and inflammatory cytokines is complex and still remains to be fully elucidated. In experimental animals and humans, TNF-α is involved in inflammatory injury induced by I/R. Recently, it was reported that a brief episode of focal cerebral ischemic injury can stimulate TNF-α messenger ribonucleic acid (mRNA) expression in adult rats (16). Work with cultured hepatocytes indicates that TNF-α or IL-1β are effective stimulators of inducible nitric oxide synthase (iNOS) mRNA expression; levels of these cytokines correlated with NO release into the culture medium (17). However, the effects of NO on proinflammatory cytokine production are still poorly defined. In the present study, we tested the hypothesis that endogenous NO down-regulated early-response cytokine generation, thereby reducing reperfusion injury.

Address reprint requests to Patrick Y-K Wong, PhD, Department of Cell Biology, UMDNJ-School of Osteopathic Medicine, 2 Medical Center Drive, Stratford, NJ 08084.

Abbreviations: HI/R, hepatic ischemia-reperfusion; ALT, alanine aminotransferase; NO, nitric oxide; TNF-α, tumor necrosis factor-α, IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; L-NAME, N^W-nitro-L-arginine methyl ester, GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

MATERIALS AND METHODS

Fischer 344 rats (male, 275–300 g body weight) were purchased from Taconic Farm (Germantown, NY). The animals had free access to food (Purina Rodent chow J001) and water. The experimental protocols followed the criteria of the Public Health Service "Guide for the care and use of laboratory animals" and were approved by the Institutional Animal Care and Use Committee.

Materials

Alanine aminotransferase (ALT) activity was measured with a Sigma diagnostic kit. Nitrate reductase (from *Aspergillus* species), *o*-dianisidine, β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH), N^{w}-nitro-L-arginine methyl ester (hydrochloride) (L-NAME), and sodium nitrite were purchased from Sigma (St. Louis, MO). The RNA Stat-60 reagent, SuperScript II, Taq DNA polymerase, and GelMarker were purchased from GIBCO BRL (Gaithersburg, MD) and Tel-Test, Inc. (Friendswood, TX), respectively. Enzyme-linked immunosorbent assay (ELISA) kits for IL-1β and TNF-α were purchased from BioSource (Camarillo, CA).

Experimental protocol

The experimental protocol for partial no-flow hepatic ischemia has been well established and previously described (2, 7). Briefly, under pentobarbital anesthesia (60 mg/kg i.p.), the trachea was cannulated (PE-240) to maintain a patent airway. A polyethylene catheter (PE 50) was advanced into the right common carotid artery and connected to a pressure transducer (Gould Statham P231D) for recording blood pressure on a Grass Model 7D Polygraph (Quincy, MA). After performing the laparotomy, the relevant branches of the hepatic artery and portal vein, supplying the left lateral and median lobes of the liver, were occluded with an atraumatic Glover bulldog clamp for 30 min. The remaining caudal 3 lobes retained an intact arterial blood supply and portal venous outflow, preventing the development of intestinal venous hypertension. Reperfusion was initiated by removal of the clamp; subsequently, the animal received 1 mL of sterile saline i.p., and the adominal incision was closed with 4-0 silk and wound staples. Group IV rats received L-NAME (10 mg/kg i.v. through the penile vein) 10 min before the start of reperfusion (I/R + L-NAME), whereas group III rats were given the equivalent volume of 0.9% saline (I/R + saline). Animals in the sham control group (group I) were subjected to the identical surgical operation without occlusion of the vessel supply to the left and median lobes of liver. The animals in group II were subjected to sham operation without I/R and injected L-NAME at a dose of 10 mg/kg iv (sham + L-NAME). Blood samples were obtained at 90 min and 4 h of reperfusion (or injection of L-NAME for group II) for determination of ALT activities, nitrite/nitrate concentration (as an index of NO production), plasma TNF-α and IL-1β concentrations, and circulating leukocyte counts. Biopsies of the ischemic lobes of the liver were taken at 4 h of reperfusion for extraction of total RNA and measurement of superoxide generation.

ALT activity

Plasma ALT activities, an index of liver injury, were measured with a Sigma test kit, DG 159-UV, and expressed as IU/L.

Counting of circulating leukocytes

Citrate-anticoagulated blood samples were obtained after 4 h of reperfusion. Fifty microliters of each sample was diluted 20-fold with 1% acetic acid solution to lyse red cells. Leukocytes were counted by light microscopy using a hemocytometer.

Plasma nitrite/nitrate assay

Plasma nitrite/nitrate was measured by the nitric oxide analyzer (NOA, model 270B, Sievers Instruments, Denver, CO) (2, 7). The NOA measures NO in biological fluids by a modified gas-stripping technique with high sensitivity (<10 picomol/mL of solution). After centrifugation of blood samples (1000 *g* for 5 min), 100 μl of each plasma sample was incubated in the presence of nitrate reductase (0.05 U/mL) and nicotinamide adenine dinucleotide phosphate (NADPH) (0.1 mM) at 37°C for 15 min to convert all the nitrate to nitrite. Subsequently, 20 μL of sample was injected into the purge vessel of the NOA, which contained 2 mL of 1% sodium iodide in acetic acid to convert the nitrite to NO gas. A stream of nitrogen was passed through the purge vessel under vacuum to eliminate any oxygen in the vessel. The amount of nitrite was calculated from a standard curve of sodium nitrite (0–400 picomoles) prepared for each run of assay tubes. Linear regression analysis of the data obtained with the standard concentrations routinely yielded a significant correlation.

Superoxide assay

Superoxide anion production in the liver samples from ischemic and non-ischemic lobes was measured by using the method of Cherry et al. (18). Briefly, tissue samples (70–180 mg) were incubated in Krebs-bicarbonate buffer (pH 7.4) consisting of (mM): NaCl (118), KCl (4.7), $CaCl_2$ (1.5), $NaHCO_3$ (25), $MgSO_4$ (1.1), KH_2PO_4 (1.2), and glucose (5.6). The tissues were gassed with 95% O_2, and 5% CO_2 for 30 min and placed in plastic scintillation vials containing 0.25 mM lucigenin in 1 mL of Krebs-bicarbonate buffer containing HEPES (pH 7.4). The chemiluminescence elicited by superoxide in the presence of lucigenin was measured by using a Mark 5303 scintillation counter (TmAnalytic, Elk Grove Village, IL). After 3 min of dark adaptation, vials containing only the cocktail (blanks) were counted 3 times for 6 s each time. The tissue samples were subsequently added to vials, allowed 3 min of dark adaptation, and counted twice (6 s each time).

Reverse transcription polymerase chain reaction (RT-PCR) amplification of mRNA

Liver samples were snap frozen in liquid nitrogen and stored at –70°C until analysis. Total cellular RNA was isolated by homogenizing tissues with a Polytron homogenizer in RNA Stat-60 reagent (Tel-Test, TX). Total RNA was extracted by chloroform and then centrifuged at 12,000 *g* for 15 min at 4°C. The RNA was precipitated by isopropanol and the pellet dissolved in diethyl pyrocarbonate water (Sigma, St. Louis, MO). Total RNA concentration was determined by spectrophotometric analysis at 260 nm, and 4 μg of total RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) in 30 μL of reaction mixture containing Superscript II (GIBCO BRL, Gaithersburg, MD), deoxynucleotide triphosphate (dNTP) and oligo (dT) 12–18 primers. The cDNA was amplified by using specific primers with a Perkin-Elmer DNA Thermal Cycler 480. The amplification mixture contained 1 μL of 15 μM forward primer, 1 μL of 15 μM reverse primer, 5 μL of 10 × buffer, 1.5 μL of 50 mM Mg^{2+}, 5 μL of the reverse transcribed cDNA samples, and 1 μL of Taq polymerase. Primers were designed from the published cDNA sequences by using the Oligo Primer Detection Program. The cDNA was amplified after determining the optimal number of amplification cycles within the exponential amplification phase for each primer set. Samples were denatured at 94°C for 5 min followed by 20 cycles for GAPDH or 25 cycles for iNOS and IL-1β. Each cycle consisted of 94°C for 45 s, 60°C for 60 s, and 72°C for 90 s. Then samples were incubated at 72°C for 6 min. Samples of TNF-α cDNA were amplified by using 31 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s. After amplification, samples (5 μL) were separated on a 2% agarose gel containing 0.5 μg/mL (0.003%) of ethidium bromide, and bands were visualized and photographed by ultraviolet transillumination. The size of each PCR product was determined by comparison with a standard DNA size marker. Semiquantitative assessment of gene expression was performed using the Image Master VDS program (Pharmacia Biotech). The designed primer sequences are shown below:

	Sense primer	Antisense primer
GAPDH	5'GGT GAA GGT CGG TGT CAA CGG ATT 3'	5'GAT GCC AAA GTT GTC ATG GAT GAC C3'
TNF-α	5'ACC ACA GAA AGA TGC TGA GGT TGG3'	5'ATG ATC CTG GAA GGG GAC AAA GGC3'
IL-1β	5'CTT CCT TGT GCA AGT GTC TGA AGC 3'	5'AAG AAG GTG CTT GGG TCC TCA TCC3'
iNOS	5'AGA AGC AGA ATG TGA CCA TCA TGG ACC ACC3'	5'AGC ACA GAA GCA AAG AAC ACC GCT TTC ACC 3'

Enzyme-linked immunosorbent assay (ELISA) for plasma TNF-α and IL-1β

Immunoreactive TNF-α and IL-1β were measured with ELISA kits purchased from BioSource (Camarillo, CA). Plasma was separated by centrifugation at 1000 *g* for 5 min at 4°C and stored at –70°C until the ELISA. The ELISA procedure for measurement of the cytokines was conducted according to the instructions of the manufacturer. Duplicate samples and standards were included in each assay. Based on the kit instructions provided by the manu-

facturer, the minimum detectable concentrations of raTNF-α and raIL-1β are 0.7 and 3 pg/mL, respectively.

Statistical analysis

Data are expressed as the mean ± SEM. Statistical significance for multigroup comparisons was determined using one-way analysis of variance (ANOVA; Sigma Stat Program) for multiple group comparisons with repeated measurement. If a significant F value was obtained, the group means were analyzed by using the Bonferroni multiple comparison test where $P < 0.05$ was considered statistically significant.

RESULTS

Plasma ALT activity

When the left and median lobes of the liver were made ischemic for 30 min, significant hepatic injury occurred during the first 4 h of reperfusion reflected by the pronounced elevation of plasma ALT activity, which averaged more than 30-fold higher than the sham control group (2080 ± 99 U/L vs. 67 ± 13 U/L). Administration of L-NAME (10 mg/kg iv, 10 min before reperfusion) exacerbated the reperfusion injury as judged by the further rise of plasma ALT activity (4982 ± 534 U/L) at 4 h of reperfusion ($P < 0.05$ relative to I/R + saline group) (Fig. 1). There was no statistically significant difference ($P < 0.05$) between sham control and sham control + L-NAME rats 4 h after injection of saline or L-NAME.

Superoxide generation

The results of assays to evaluate hepatic superoxide generation are shown in Fig. 2. At 4 h of reperfusion, superoxide generation in the ischemic lobes of rats treated with L-NAME was significantly greater (3307 ± 397 cpm/mg tissue) than that in samples from group III rats (1862 ± 111 cpm/mg tissue; group IV vs. group III, $P < 0.05$) and far exceeded the levels in liver samples from sham controls (903 ± 81 cpm/mg tissue) and sham control + L-NAME (781 ± 64 cpm/mg tissue; group III vs. groups I and II, $P < 0.05$).

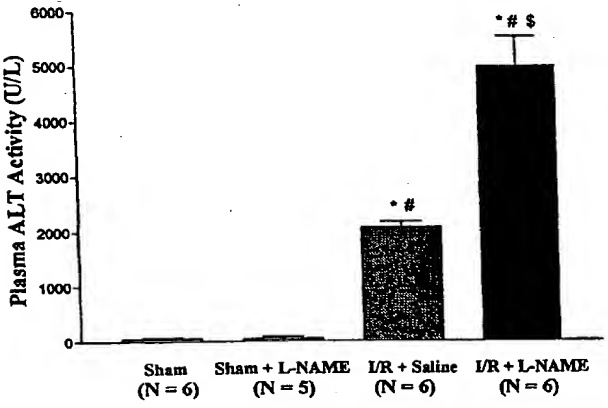


FIG. 1. **Effects of 30 min of hepatic ischemia followed by 4 h of reperfusion on plasma alanine aminotransferase (ALT) activities in rats administered L-NAME (10 mg/kg iv 10 min before reperfusion) (group IV) or saline (group III).** Data represent means ± SE of 5–6 animals. *$P < 0.05$ compared with sham control (group I); #$P < 0.05$ compared with sham-operated group (group II); \$$P < 0.05$ compared with I/R + saline animals (group III).

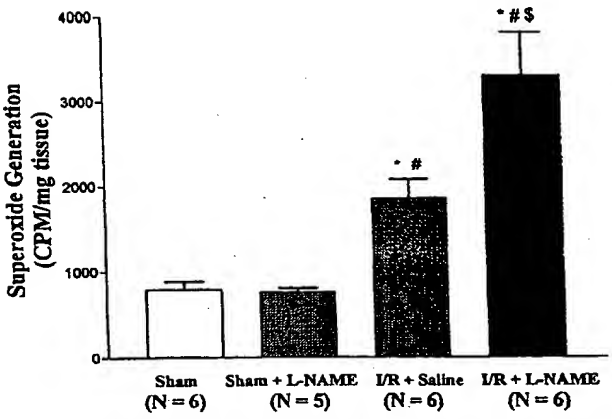


FIG. 2. **Effects of 30 min of hepatic ischemia followed by 4 h of reperfusion on superoxide generation in the ischemic lobes of liver of rats administered L-NAME (10 mg/kg iv 10 min before reperfusion) (group IV) or saline (group III).** Data represents means ± SE of 5–6 animals. *$P < 0.05$ compared with sham control (group I); #$P < 0.05$ compared with sham-operated group (group II); \$$P < 0.05$ compared with I/R + saline animals (group III).

Circulating leukocytes

An elevation of circulating leukocytes, suggesting a systemic inflammatory response, was observed after hepatic reperfusion injury. Group III rats exhibited a 1.81-fold increase in circulating leukocytes (5608 ± 542 cells/mm^3) compared with group I animals (3098 ± 265 cells/mm^3), and a 1.52-fold increase compared with group II (3685 + 313 cells/mm^3) at 4 h of reperfusion. The manifestation of this inflammatory response was potentiated in group IV rats (7950 ± 610 cells/mm^3, $P < 0.05$ vs. group III). There was no statistically significant difference ($P < 0.05$) between groups I and II (Fig. 3).

Plasma nitrite/nitrate

There was no statistically significant difference in plasma nitrite/nitrate levels between sham control group (9.9 ± 1.2

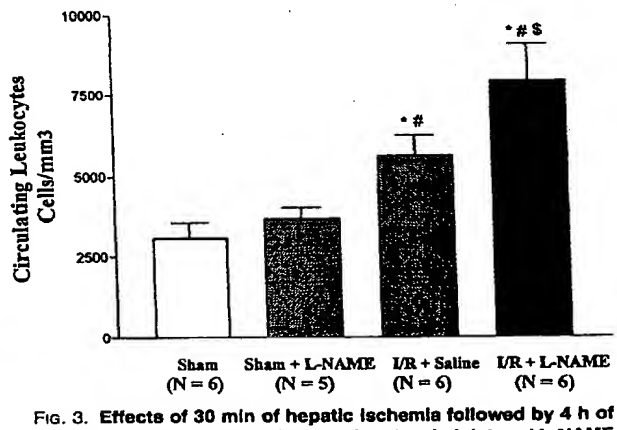


FIG. 3. **Effects of 30 min of hepatic ischemia followed by 4 h of reperfusion on circulating leukocytes in rats administered L-NAME (10 mg/kg iv 10 min before reperfusion) (group IV) or saline (group III).** The results are presented as means ± SE of 5–6 animals. *$P < 0.05$ compared with sham control (group I); #$P < 0.05$ compared with sham-operated group (group II); \$$P < 0.05$ compared with I/R + saline animals (group III).

μmol/L), sham control + L-NAME (8.17 ± 1.7 μmol/L), and I/R + L-NAME rats (7.5 ± 1.0 μmol/L). Plasma nitrite and nitrate levels of I/R rats at 4 h of reperfusion were 39.4 ± 4.0 μmol/L ($P < 0.05$ vs. groups I, II, and IV) (Fig. 4).

Plasma TNF-α concentrations

The effects of I/R on plasma TNF-α concentrations were determined at 90 min (Fig. 5) and at 4 h (Fig. 6) of reperfusion, respectively. Immunoreactive TNF-α in group I and II animals averaged 2.4 ± 0.7 and 4.4 ± 5.1 pg/mL 90 min after injection of saline or L-NAME, respectively. Compared with these mean values, levels in group III rats (102.9 ± 19 pg/mL), there were 42-fold (vs. group I) and 23-fold (vs. group II). Although plasma TNF-α levels of group IV rats (I/R + L-NAME) tended to be higher (135 ± 16 pg/mL) than the levels in group III rats at 90 min of reperfusion, this change failed to reach statistical significance (Fig. 5).

At 4 h of reperfusion, mean plasma TNF-α concentration of group III rats (10.5 ± 0.8 pg/mL) was 10-fold lower than that observed at 90 min of reperfusion (102.9 ± 19 pg/mL) but still markedly elevated compared with group I animals (1.6 ± 0.5). At 4 h of reperfusion, plasma TNF-α concentration of group IV rats (22 ± 4.12 pg/mL) was significantly elevated compared with group III. Plasma TNF-α levels in group II were undetectable 4 h after administration injection of L-NAME to sham-operated rats (Fig. 6).

Plasma IL-1β concentrations

At 90 min of reperfusion, plasma IL-1β was not detectable in sham control animals compared with 116 ± 16 pg/mL in group III and 441 ± 113 pg/mL in group IV. A 4-fold increase was observed in group IV vs. group III (Fig. 5). A similar elevation (5.8-fold) was observed in group IV (415 ± 90 pg/mL) vs. group III (71 ± 8 pg/mL) at 4 h of reperfusion ($P < 0.05$) (Fig. 6). Plasma IL-1β levels in groups I and II were undetectable 90 and 240 min after injection of saline or L-NAME, respectively.

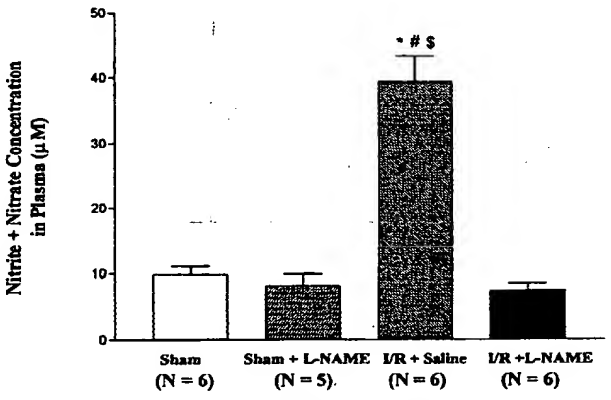


FIG. 4. **Effects of 30 min of hepatic ischemia followed by 4 h of reperfusion on plasma nitrite/nitrate concentration in rats administered L-NAME (10 mg/kg iv 10 min before reperfusion) (group IV) or saline (group III).** Data represent means ± SE of 5–6 animals. *$P <$ 0.05 compared with sham control (group I); #$P < 0.05$ compared with sham-operated group (group II); $$P < 0.05$ compared with I/R + L-NAME animals (group IV).

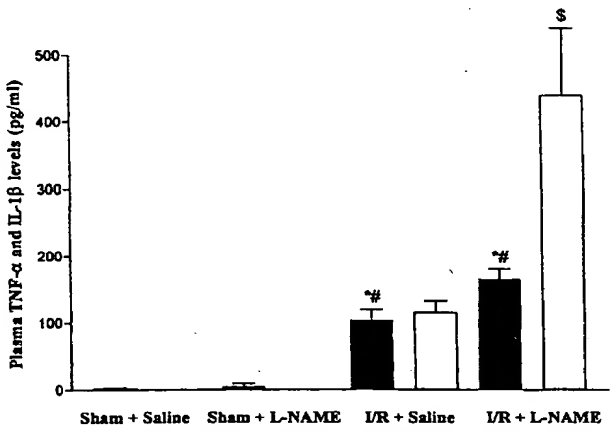


FIG. 5. **Effects of 30 min of hepatic ischemia followed by reperfusion on plasma TNF-α and IL-1β concentrations measured with ELISA kits (BioSource) at 90 min in rats of the experimental groups.** The results are presented as means ± SE of 5–6 animals. *$P <$ 0.05 compared with the sham-operated group (group I); #$P < 0.05$ compared with sham-operated animals plus 1-NAME (group II); $$P < 0.05$ compared with I/R + saline animals (group III). Plasma IL-1β levels in group I and II rats were undetectable. Solid bars, TNF-α; open bars, IL-1β.

iNOS, TNF-α, and IL-1β mRNA expression

Total mRNA was extracted from the livers of sham controls and from ischemic lobes of livers of groups III and IV at 4 h of reperfusion. Expression of iNOS, TNF-α, and IL-1β mRNA was studied by RT-PCR amplification after separating products by gel electrophoresis (Fig. 7a). A semiquantitative analysis of

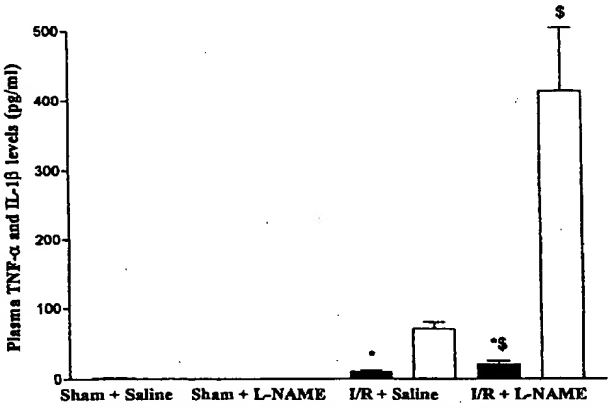
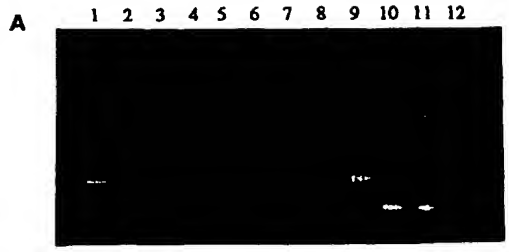


FIG. 6. **Effects of 30 min of hepatic ischemia followed by reperfusion on plasma TNF-α and IL-1β concentrations measured with ELISA kits (BioSource) at 4 h of reperfusion in rats of the experimental groups.** The results are presented as means ± SE of 5–6 animals. *$P <$ 0.05 compared to group I; $$P < 0.05$ compared with I/R + saline animals (group III). Plasma TNF-α levels in group II and IL-1β levels in group I and group II rats were undetectable. Solid bars, TNF-α; open bars, IL-1β.



Lane 1, 5, 9: GAPDH; 2, 6, 10: iNOS; 3, 7, 11: TNF-α; 4, 8, 12: IL-1β.

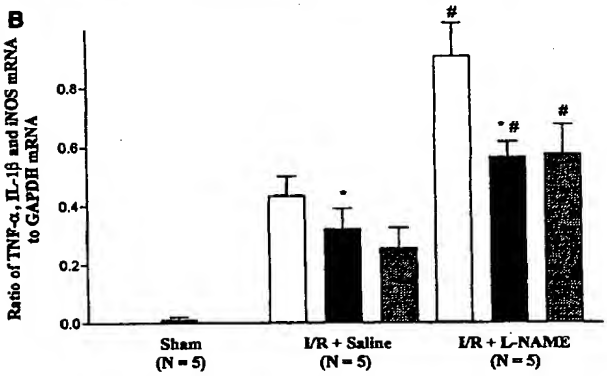


FIG. 7. **(A) Ethidium bromide-stained agarose gel showing PCR products from amplified rat hepatic RNA.** HI/R was conducted as described in Materials Methods, and the ischemic liver lobes were obtained at 4 h of reperfusion. RNA was extracted from hepatic tissue and reverse transcribed and amplified using primers selected from published cDNA sequences. Lanes 1–4, sham control (group I); lanes 5–8, I/R + saline (group III); lanes 9–12, I/R + L-NAME (group IV). Lanes 1, 5, 9, GAPDH; lanes 2, 6, 10, iNOS; lanes 3, 7, 11, IL-1β; lanes 4, 8, 12, TNF-α. A semiquantitative analysis of the results of RT-PCR was performed with the Image Master VDS program (Pharmacia Biotech). With this computer program, the electrophoretic bands were normalized for GAPDH gene expression. The normalized ratios for TNF-α, IL-1β, and iNOS mRNA expression in the experiments were averaged, and the results are shown in Fig. 7B. Data represent means ± SE of 5 animals. *$P < 0.05$ compared with sham control; #$P < 0.05$ compared with I/R + saline animals. Open bars, ratio of TNF-α; solid bars, ratio of IL-1β; hatched bars, ratio of iNOS to GADPH.

RT-PCR was performed with the Image Master VDS program (Pharmacia Biotech) and normalized by assessment of the expression of GAPDH. Applying these procedures to liver extracts from sham controls, we were unable to detect TNF-α and iNOS mRNA expression and found a minimal degree of IL-1β mRNA expression. However, evidence of expression of iNOS, TNF-α, and IL-1β genes was obtained when the RT-PCR approach was applied to liver extracts from group III and IV rats. An episode of I/R caused significant increases in iNOS (comparative densitometry ratio of 0.26, relative to GAPDH), TNF-α (0.44, relative to GAPDH) and IL-1β (0.32, relative to GADPH) gene expression in the ischemic lobes. Administration of L-NAME to rats subjected to HI/R resulted in an enhancement of gene expression for iNOS (0.58, relative to GAPDH), TNF-α (0.91, relative to GAPDH) and IL-1β (0.56, relative to GAPDH) (Fig. 7b).

DISCUSSION

An extensive literature indicates that NO is a key regulator of vascular tone and exerts a wide array of immunoinflammatory effects. In the vasculature, physiological concentrations of endogenous NO contribute to the regulation of vascular tone and leukocyte-endothelial interactions (8, 19), with influences on platelet aggregation and neutrophil infiltration (19, 20). Excessive production of NO is likely to be detrimental because high concentrations of NO are cytotoxic. Mechanisms by which NO could produce cell injury include inhibition of iron-containing enzyme activities, production of the reactive oxidant peroxynitrite, disruption of protein function by nitrosylation of amino acids, and alteration of DNA synthesis. Our previous results indicated that administration of L-NAME to rats subjected to HI/R attenuated NO and peroxynitrite production. However, the decrease in NO generation exacerbated leukocyte infiltration into the ischemic lobe of liver (2, 7) by enhancing adhesion molecular expression (8). In the present study we tested the hypothesis that endogenous NO down-regulated early-response cytokine generation, thereby reducing reperfusion injury.

The data presented in this study indicated that inhibition of NO synthesis by L-NAME exacerbated ischemia-reperfusion injury as evidenced by a 2.4-fold increase in plasma ALT activity (Fig. 1) and 1.8-fold increase of superoxide generation (Fig. 2). The mechanism(s) responsible for the exacerbation of ischemia-reperfusion injury by L-NAME may be, in part, augmentation of TNF-α and IL-1β generation at the levels of protein synthesis (Figs. 5 and 6) and mRNA expression (Fig. 7).

A recent conceptual advance is the recognition that the severity of tissue injury is not determined solely by exogenous factors, e.g., a microbial agent liberating lipopolysaccharide (LPS), but rather is largely a consequence of the host's overwhelming immunoinflammatory response (21). It appears that the host's endogenous mediators provoke multiple acute pathological reactions formerly attributed to endotoxin, e.g., hypotension and inflammatory cell activation. The symptoms of infection are mainly caused by cytokines, notably TNF-α and IL-1β, released from inflammatory cells. Furthermore, release of one cytokine often triggers secretion of additional cytokine(s) on the cytokine cascade, which can further amplify the inflammatory responses (21, 22).

The literature indicates that TNF-α and IL-1β are powerful stimuli for endothelial cell activation, augmenting inducible NO synthase (iNOS) expression, and producing pathological levels of NO (17, 23, 24). As single agents, TNF-α or IL-1β proved to be effective stimulators for iNOS mRNA expression in the cultured hepatocyte, correlating well with elevated NO release (17). On the other hand, studies on NO involvement in regulation of TNF-α and IL-1β production are rare, and the results are conflicting. Treatment with the NOS inhibitor L-NMMA suppressed production of IL-1, IL-2, IL-6, and IFN-γ, and increased the secretion of IL-10 in the mouse footpad model (25) and inhibited IL-8 and IL-6 production *in vitro* (26). In contrast, levels of IL-8 and IL-6 in bronchoalveolar

lavage fluid decreased when patients with ARDS were treated with NO by inhalation (22).

In the HI/R model, we were unable to detect levels of mRNA expression of cytokine-induced neutrophil chemoattractant (CINC) and IL-10 in the ischemic lobes of liver at 4 h of reperfusion (data not shown). However, the generation of TNF-α and IL-1β was significantly altered (Figs. 5–7). Our results indicated that HI/R induced the generation of TNF-α and IL-1β based on mRNA expression and protein synthesis. The generation of TNF-α and IL-1β in response to HI/R was enhanced by the administration of L-NAME. However, no statistically significant differences were found between group I and group II rats for any of the parameters monitored, indicating that reperfusion injury was a prerequisite for effects of L-NAME on early cytokine release.

Administration of L-NAME to rats with HI/R was associated with a significant increase in iNOS mRNA expression in the ischemic lobes of liver at 4 h of reperfusion (Fig. 7B), although mean plasma NO concentration was about 5-fold lower than the mean of rats subjected to HI/R (Fig. 4). These results are consistent with the reports that negative feedback regulation by NO plays an important role in iNOS gene expression (27, 28).

Several mechanisms could account for the observed effects of NO synthase inhibition on early-response cytokine generation. First, NO may decrease the density of ICAM-1 and P-selectin expression on endothelial cells (8, 19) and CD11/CD18 expression on neutrophils (13, 20). Second, in vascular smooth muscle cells, NO can directly activate guanylate cyclase and lead to cyclic guanosine monophosphate (cGMP) generation, which causes vasorelaxation (8, 11, 29). Futhermore, NO increases cardiac output and stroke volume (8, 29). Both the reduction of vascular resistance and the elevation of cardiac output could improve tissue perfusion and attenuate the inflammatory response. Third, it is tempting to speculate that NO may exert an inhibitory effect on TNF-α and IL-1β generation in part at the transcriptional level. Electrophoretic mobility shift assays indicated that NO suppresses VCAM-1 gene transcription by inhibiting NF-κB (30). These reports suggested that NO suppresses TNF-α and IL-1β gene transcription by inhibiting the activation of the transcription factor NF-κB. Because the activation of NF-κB may also depend on the presence of reactive oxygen species, NO may interfere with NF-κB activation by scavenging and inactivating superoxide anion.

The present study also demonstrated an increase in the total number of circulating leukocytes in rats subjected to HI/R + L-NAME (Fig. 3), in agreement with a report by Geffner et al. (31). The mechanism for the enhancement of circulating leukocytes by the NOS inhibitor probably involves increased generation of TNF-α and IL-1β, both of which enhance the production and release of leukocytes from the bone marrow.

Some evidence indicates a lack of correlation between plasma levels of TNF-α and IL-1β and the clinical outcome of patients (32). An explanation for the poor clinical correlation may be due to the protective action of certain cytokine antagonists, such as IL-1ra and sTNF-R (13, 33). These endogenous antagonists along with anti-inflammatory cytokines, such as IL-10, are produced systemically after the initial insult and may modulate plasma TNF-α and IL-1β levels. In addition, a gradient in the levels of cytokines has been found between the original inflamed organs and peripheral blood flow (34).

Our studies raise the question of whether the increase of endogenous NO during various inflammatory states (with the exception of septic shock) is a compensatory response to the pathological stimuli or a deleterious reaction? Our results presented here suggest that in the early acute phase of inflammation induced by hepatic I/R, a compensatory increase in NO production repressed TNF-α and IL-1β gene expression in the ischemic lobes and attenuated plasma levels of TNF-α and IL-1β. Therefore, the net effect of endogenous NO was to ameliorate hepatic injury in the early phase of ischemia-reperfusion.

ACKNOWLEDGMENT

This work was supported by a Grant-In-Aid (NJ-97-GS-16) from The American Heart Association, New Jersey Affiliate, Inc. to P.L.

REFERENCES

1. Thiemermann C, Vane JR: Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharide in the rat *in vivo*. *Eur J Pharmacol* 182:591–595, 1990.
2. Liu P, Yin K, Yue G, Wong PY-K: Role of nitric oxide in hepatic ischemia-reperfusion with endotoxemia. *J Inflamm* 46:144–154, 1996.
3. Liu P, Hock EC, Nagele R, Wong PY-K: Formation of nitric oxide, superoxide and peroxynitrite in myocardia ischemia-reperfusion in rats. *Am J Physiol* 272:H2327–H2336, 1997.
4. Evans T, Carpenter A, Kinderman H, Cohen J: Evidence of increased nitric oxide production in patients with the sepsis syndrome. *Circ Shock* 41:77–81, 1993.
5. Ochoa JB, Udekwu AO, Billiar TR, Curran RD, Cerra FB, Simmons RL, Peitzman AB: Nitrogen oxide levels in patients after trauma and during sepsis. *Ann Surg* 214:621–626, 1991.
6. Petros A, Bennett D, Vallance P: Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *Lancet* 338:1558–1559, 1991.
7. Liu P, Yin K, Nagele R, Wong PY-K: Inhibition of NOS attenuates peroxynitrite generation, but augments neutrophil infiltration in hepatic ischemia-reperfusion in rats. *J Pharmacol Exp Ther* 284:1139–1146, 1998.
8. Liu P, Xu B, Hock CE, Nagele R, Sun FF, Wong PY-K: Nitric oxide modulates P-selectin and ICAM-1 mRNA expression and hemodynamic alterations in hepatic ischemia-reperfusion. *Am J Physiol* 275:H2191–H2198, 1998.
9. Minnard EA, Shou J, Naama H, Cech A, Gallagher H, Daly JM: Inhibition of nitric oxide synthesis is detrimental during endotoxemia. *Arch Surg* 129:142–148, 1994.
10. Cobb JP, Natanson C, Hoffman WD, Lodato RF, Banks S, Koev CA, Solomon MA, Elin RJ, Hosseini JM, Danner RL: N omega-amino-L-arginine, an inhibitor of nitric oxide synthase, raises vascular resistance but increases mortality rates in awake canines challenged with endotoxin. *J Exp Med* 176:1175–1182, 1992.
11. Avontuur JAM, Biewenga M, Buijk SLCE, Kanhai KLK, Bruining HA: Pulmonary hypertension and reduced cardiac output during inhibition of nitric oxide synthesis in human septic shock (case report). *Shock* 9:451–454, 1998.
12. Nast-Kolb D, Waydhas, Gippner-Steppert C, Schneider I, Trupka A, Ruchholtz S, Zettl R, Schweiberer L, Jochum M: Indicators of the posttraumatic inflammatory response correlate with organ failure in patients with multiple injuries. *J Trauma* 42:446–453, 1997.
13. Aderka D, Sorkine P, Abu-Abid S, Lev D, Setton A, Cope PA, Wallach D, Klausler J: Shedding kinetics of soluble tumor necrosis factor (TNF) receptors after systemic TNF leaking during isolated limb perfusion. *J Clin Invest* 101:650–659, 1998.
14. Foreman KE, Glovsky MM, Warner RL, Horvath SJ, Ward PA: Com-

parative effect of C3a and C5a on adhesion molecule expression on neutrophils and endothelial cells. *Inflammation* 20:1–9, 1996.
15. Amsterdam EA, Stahl GL, Pan HL, Rendig SV, Fletcher MP, Longhurst JC: Limitation of reperfusion injury by a monoclonal antibody to C5a during myocardial infarction in pigs. *Am J Physiol* 268:H448–457, 1995.
16. Feuerstein GZ, Liu T, Barone FC: Cytokines, inflammation and brain injury: role of tumor necrosis factor-alpha. *Cerebrovasc Brain Metab Rev* 6:341–360, 1994.
17. Lau KS, Nakashima O, Aalund GR, Hogarth L, Ujiie K, Yuen J, Star RA: TNF-alpha and IFN-gamma induce expression of nitric oxide synthase in cultured rat medullary interstitial cells. *Am J Physiol* 269(F):212–217, 1995.
18. Cherry PD, Omar HA, Farrell KA, Stuart JS, Wolin MS: Superoxide anion inhibits cGMP-associated bovine pulmonary arterial relaxation. *Am J Phyiol* 259:H1059–H1062, 1990.
19. Gauthier TW, Davenpeck KL, Lefer AM: Nitric oxide attenuates leukocyte-endothelial interaction via P-selectin in splanchnic ischemia-reperfusion. *Am J Physiol* 267:G562–G568, 1994.
20. Kubes P, Suzuki M, Granger DN: Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci USA* 88:4651–4655, 1991.
21. Anderson BO, Harken AH: Multiple organ failure: Inflammatory priming and activation sequences promote autologous tissue damage. *J Trauma* 30:S44–49, 1990.
22. Chollet-Martin S, Gatecel C, Kermarrec N, Gougerot-Pocidalo MA, Payen DM: Alveolar neutrophil functions and cytokine levels in patients with the adult respiratory distress syndrome during nitric oxide inhalation. *Am J Respir Clin Care Med* 153:985–990, 1996.
23. Hibbs JB, Westenfelder C, Taintor R, Vavrin Z, Kablitz C, Babanowski JP, Ward JH, Menlove RL: Evidence for cytokine-inducible nitric oxide synthesis from L-arginine in patients receiving interleukin-2 therapy. *J Clin Invest* 89:867–877, 1992.
24. Ono K, Matsumori A, Shioi T, Furukawa Y, Sasayama S: Cytokine gene expression after myocardial infarction in rat heart: possible implication in left ventricular remodeling. *Circulation* 98:149–156, 1998.
25. Ianaro A, O'Donnell CA, Di Rosa M, Liew FY: A nitric oxide synthase inhibitor reduces inflammation, down-regulates inflammatory cytokines and enhances interleukin-10 production in carrageenin-induced oedema in mice. *Immunology* 82:370–375, 1994.
26. Villarete LH, Remick DG: Nitric oxide regulation of interleukin-8 gene expression. *Shock* 7:29–35, 1997.
27. Sheehy AM, Burson MA, Black SM: Nitric oxide exposure inhibits endothelial NOS activity but not gene expression: a role for superoxide. *Am J Physiol* 274:L833–841, 1998.
28. Li S, Fan SX, McKenna TM: Role of nitric oxide in sepsis-induced hyporeactivity in isolated rat lungs. *Shock* 5:122–129, 1998.
29. Herderson J, Statman R, Cunningham JN, Chang C, Damiani P, Siconolfi A, Horovitz JH: The effects of nitric oxide inhibition on regional hemodynamics during hyperdynamic endotoxemia. *Arch Surg* 129:1271–1275, 1994.
30. Kurose I, Saito H, Miura S, Ebinuma H, Higuchi H, Watanabe N, Shigeyuki Z, Nakamura T, Takaishi M, Ishii H: CD18/ICAM-1-dependent oxidative NF-κB activation leading to nitric oxide production in rat Kupffer cells cocultured with syngeneic hepatoma cells. *J Clin Invest* 99:867–878, 1997.
31. Geffner JR, Trevani AS, de D'Elia I, Diament M, Klein D, Giordano M: Involvement of nitric oxide in the regulation of peripheral blood leukocyte counts. *J Leukoc Biol* 58:391–394, 1995.
32. Bone R: Why sepsis trials fail, a commentary. J Am Med Assoc 276:565–566, 1996.
33. Gabay C, Smith MF, Eidlen D, Arend WP: Interleukin 1 receptor antagonist (IL-1Ra) is an acute-phase protein. *J Clin Invest* 99:2930–2940, 1997.
34. Mester M, Carter EA, Tompkins RG, Gelfand JA, Dinarello CA, Burke JF, Clark BD: Thermal injury induces very early production of interleukin-1 alpha in the rat by mechanisms other than endotoxemia. *Surgery* 115:588–596, 1994.